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SPECIFICATION

# PREVENTIVE AND THERAPEUTIC COMPOSITIONS FOR DRUG-INDUCED NEPHROPATHY AND HEPATOPATHY

## Technical Field

The present invention relates to drugs for relieving drug-induced nephropathy and hepatitis.

## Background Art

The use of drugs for therapeutic and/or diagnostic purposes has increased year by year, and the drugs used have diversified. These drugs can provide us significant benefits but can also cause substantially harmful effects, especially to kidneys, due to their specific functions described below.

Kidneys weigh less than one percent of the total body weight. From a physiological viewpoint, 25 percent of the total cardiac output flows into the kidneys; 150 liters of primitive urine, up to 50 times the total blood plasma, is filtered through glomeruli per day; and final urine is made by reabsorption, secretion, and metabolism through uriniferous tubules variable in structural and functional heterogeneity. Thus, drugs or their metabolites in blood always circulate, and these substances are concentrated and metabolized in kidneys. Consequently, various highly concentrated metabolites, including original drugs, are distributed in kidneys. Kidneys are likely to be frequently and intensively exposed to drugs. Four types of drugs may induce nephropathy: antimicrobial agents, nonsteroidal agents, contrast agents, and antitumor agents.

The liver can also be easily damaged by drugs. Drug-induced hepatopathy is classified by its onset mechanism into toxic hepatopathy caused by direct attack of drugs or their intermediate metabolites to the liver, and allergic hepatopathy caused by allergic response, type IV delayed allergic response in which T cells are involved. Drug-induced hepatitis is caused by, most frequently, antibiotics, followed by drugs for the central nervous system, drugs for circulatory organs, antitumor agents, hormonal agents, diagnostic agents, etc.

Attempts have been made to relieve drug-induced disorders by using γ-globulin, cytochrome C, adenine, SH compounds, vitamin B group, etc., but they are not sufficiently effective. It is very important to clinically cure drug-induced disorders (side effects) because of the interruption of the treatment and the importance of patients' quality of life (Q.O.L.).

Disclosure of the Invention

An objective of the present invention is to drugs that effectively relieve or suppress disorders induced by various drugs, especially by antitumor agents.

The inventors have focused on the facts that proteins belonging to the midkine (MK) family such as midkine (MK) and pleiotrophin (PTN) are growth and differentiation factors with multiple functions. The functions include 1) elongation of neurite, 2) activation of fibrinolytic system, 3) strong expression in human cancerous areas, and 4) cure of wounds. Numerous studies have been performed on such proteins in order to find novel pharmaceutical effects.

Midkine was discovered as a product of the gene whose expression was induced in the early stage of the differentiation process with retinoic acid in mouse embryonic tumor cells (Kadomatsu, K. et al., Biochem. Biophys. Res. Commun., 151: 1312-1318, 1988). Pleiotrophin was discovered in the brain of a newborn rat as a heparin-binding protein with neurite elongation ability (Rauvala, H., EMBO J., 8: 2933-2941, 1989). Midkine and Pleiotrophin form a novel class of growth and differentiation factors as heparin-binding proteins. They exhibit 45% homology and are collectively called the MK family (Muramatsu, T., Int. J. Dev. Biol., 37: 183-188, 1993; Muramatsu, T., Develop. Growth & Differ. 36(1): 1-8, 1994). Midkine and Pleiotrophin each exhibits a specific expression pattern in development processes, and is expected to be involved in important physiological activation in differentiation.

The inventors found that MK inhibits cell death caused by antitumor agents *in vitro* and that MK gene relieves disorders induced by an antitumor agent from the results of an experiment in which an antitumor agent was administered to knockout mice in which MK gene

was functionally destroyed. The inventors also found that administering MK or PTN to wild mice relieves the disorders caused by antitumor agents, to complete the invention. The present invention encompasses each invention described in the claims.

In this invention, knockout mice provided an opportunity to investigate how MK gene in the living body fights against disorders caused by drugs and to analyze how each knockout mouse responds to the forced administration of MK at the individual level. Details of MK's function and mechanism are presently not clear. If MK functions as a trigger protein for the functional cascade of cytokines or growth factors, a very small amount of MK is presumably needed, and the use of knockout mice becomes more important. Recently, a cell surface receptor specifically binding to MK with high affinity (molecular weight 250 + kDa) has been discovered. Its characteristics imply that autocrine stimulated by MK in tumor cell proliferation could be mediated by the receptor and would activate the JAK/STAT pathway (Edward, A. R. et al., J. Biol. Chem. 273: 3654-3660, 1998).

To clarify the relationship between MK and ontogenesis, homozygous MK gene-knocked out mice in which parts of exon 2 and exon 3 are damaged as illustrated in Figure 1 were prepared (Biochemistry 7, Heisei 8: Volume 68, pp. 1239, 4-P-1244). Those knockout mice did not die during the fetal period and weighed significantly less than heterozygous or wild types (Biochemistry 7, Volume 68, pp. 1239, 4-P-1244, 1996).

Antitumor agents were administered to the knockout mice (simply referred as knockout mice) and wild mice. Survival rate, blood urea nitrogen (BUN) level, and creatinine level of each mouse were compared as indices of disorders after the administration to monitor the ability of MK gene in the living body to relieve disorders caused by antitumor agents. BUN and creatinine levels can be used as indices of functional disorders in kidneys because urea is accumulated in blood due to the reduced renal excretory ability.

In this invention, cisplatin was administered to the knockout mice and wild mice, then BUN level and survival rate were compared. The BUN level of the knockout mice were significantly higher than that of the wild mice. The survival rate of the knockout mice also

differed significantly from that of wild mice. The death rate of knockout mice increased by the seventh day after the administration. The rate of abnormal BUN levels in knockout mice that had been forcedly administered MK was significantly lower than that in the group that had been administered physiological saline by the third day after the administration. The effectiveness of MK in suppressing renal cell disorders caused by cisplatin was confirmed by conducting an experiment *in vitro* using human infantile renal cancer cell lines. An experiment using the wild mice revealed that MK relieved acute hepatopathy due to carbon tetrachloride, and that both PTN and MK effectively suppress nephropathy caused by cisplatin.

These results indicate that proteins of this invention belonging to the MK family effectively relieve or suppress drug-induced nephropathy and hepatopathy.

MK protein (simply referred to as MK) used as an effective ingredient of the pharmaceutical composition of the invention is described in the following references (human MK gene, unexamined published Japanese patent application (JP-A) No. Hei 5-91880; sequences of the human MK gene and protein, JP-A No. Hei 6-217778; MK protein, JP-A No. Hei 5-229957; Muramatsu, T., Develop. Growth & Differ. 36(1), 1-8, 1994). PTN protein used as an effective ingredient of the pharmaceutical composition of the invention is described in the following references (Muramatsu, T., Develop. Growth & Differ. 36(1), 1-8, 1994; Andreas, K. et al., Critical Reviews in Oncogenesis, 6(2): 151-177, 1995). The proteins belonging to the MK family and used as effective ingredients of the pharmaceutical composition of this invention include natural proteins derived from humans, mice, or other mammals, or artificial proteins manufactured by chemical synthesis or genetic engineering. Also, the proteins of the invention that belong to the MK family include proteins or polypeptides which do not cause any changes of the above-described biological activities and differ from proteins derived from nature in the number or the sequences of amino acids. Specifically, the present invention includes proteins corresponding to natural proteins in which the amino acid sequence of natural proteins is partially deleted or replaced by other amino acids, or other amino acids or

polypeptide of different length are inserted or added. Amino acids to be replaced or inserted are not limited to natural types.

The expression system using *E. coli* (Studier, F. W. & Moffatt, B. A., J. Mol. Biol. 189: 113-130, 1989; Studier, F. W. et al., Meth. Enzymol. 185: 60-89, 1990) or the expression system using baculovirus (O'Reilly, D. R. et al., Baculovirus Expression Vectors, A Laboratory Manual, Oxford University Press, 1992, Ausubel, F. M. et al. eds., Current Protocol in Molecular Biology, Unit 16.11, Wiley Interscience, 1994) can be used to obtain the MK family proteins that are effective ingredients of the invention using genetic engineering techniques. The inventors employed the expression system using methyl alcohol dependent yeast *Pichia pastoris* to obtain the MK proteins (refer to JP-A No. Hei 7-255354).

The pharmaceutical composition of the invention contains the MK family protein in an amount effective to prevent or treat nephropathy or hepatopathy caused by drugs. The effective ingredients of the invention can be prepared in a desirable dosage form by mixing with usually used pharmaceutically acceptable carriers, vehicles, diluents, preservatives, stabilizers, buffers, etc.

The pharmaceutical composition of the invention can be administered orally or parenterally. Dosage forms for oral administration include tablets, granules, and capsules. Dosage forms for parenteral administration include injection, suppositories, or percutaneous agents, which are administered intravenously, subcutaneously, intramuscularly, or intraperitoneally.

Physiologically active peptides such as MK or PTN are rapidly digested by protease in digestive tracts in general when they are administered orally. To stabilize MK or PTN *in vivo*, a hybrid MK or hybrid PTN should be prepared by binding it to water-soluble macromolecules (for example, polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP)). Hybrid constructions of IL-6, TNF-$\alpha$, etc. have been attempted, and the function has been enhanced by selecting the most suitable hybrid condition (Tsutsumi, Y. et al., Br. J. Cancer. 74: 1090-1095; Tsutsumi, Y. et al., Thoromb. Haemostasis, 77: 168-173, 1997; Tsutsumi, Y. et al., J. Control Release, 33: 447-451, 1995).

The MK family proteins that are effective ingredients of the

pharmaceutical compositions of the invention vary depending on the dosage of the causative drug, severity of nephropathy or hepatopathy, age, sex, and weight of the patient when used to prevent or treat nephropathy or hepatopathy caused by drugs. The proteins of the invention can be administered once or several times at a dosage of 1 $\mu$g/kg to 100 mg/kg of body weight per day.

Brief Description of the Drawings

Figure 1 shows the mutant chromosome of the knockout mouse in which parts of exons 2 and 3 of MK gene of 129/Sv mice was destroyed.

Figure 2 shows the survival rate of 129/Sv MK knockout mice and that of wild mice after the administration of cisplatin.

Figure 3 shows blood urea nitrogen of 129/Sv MK knockout mice and that of wild mice on the day of administration and on the third and fifth days after the administration of cisplatin.

Figure 4 shows the frequency of abnormal blood urea nitrogen of 129/Sv MK knockout mice on the day of administration and on the third and fifth days after 14 mg/kg cisplatin was administered and MK or physiological saline absorbed capsules for sustained release were intraperitoneally implanted.

Figure 5 shows the serum GOT of mice suffering from acute hepatopathy caused by carbon tetrachloride when various concentrations of MK or physiological saline were administered to the mice.

Figure 6 shows the serum GTP of the same mice as in Figure 5.

Figure 7 shows the survival rate of G401 cells where 2 x $10^4$ cells/well were cultured in the medium alone, the medium with 2 $\mu$ g/ml of MK, or the medium with 10 $\mu$g/ml of MK, and treated with briplatin (cisplatin) during the culture.

Figure 8 shows the survival rate of G401 cells (6 x $10^4$ cells/well) after the same treatment as in Figure 7.

Figure 9 shows BUN levels before and after the administration of briplatin (cisplatin) to mice during the administration of PTN or physiological saline.

Figure 10 shows the serum creatinine of the mice in Figure 9.

Best Mode for Implementing the Invention

The present invention is illustrated in detail below with reference to examples, but is not to be construed as being limited thereto.

Example 1: Effect of MK on relieving nephropathy *in vivo*

The 129/Sv knockout mice in which parts of exons 2 and 3 of MK genes were destroyed as illustrated in Figure 1 were used (Biochemistry 7, Volume 68, pp 1239, 4-p-1244, 1996). Figure 2 shows the survival rate of the 129/Sv MK gene-knocked out mice and that of wild mice after the intraperitoneal administration of 14 mg/kg cisplatin (product name, briplatin, Bristol Myers Squibb Company). Figure 3 shows BUN levels 0, 3, and 5 days after the administration of the cisplatin. Cisplatin was selected because it is the fastest, most effective, and most common antitumor agent against solid tumors. Moreover, the side effect of cisplatin is nephropathy such as acute renal failure and MK is expressed only in kidneys in adult mice.

The Student t test revealed that the BUN levels of knockout mice were significantly higher than that of wild mice, as indicated in Figure 3.

Figure 4 shows the frequency of abnormal blood urea nitrogen of mice at zero, three, and five days after the administration of 14 mg/kg of cisplatin; 207 mg of MK-containing sustained release capsules were intraperitoneally implanted in seven mice of the MK administration group , and 207 mg of physiological saline-containing sustained release capsules were intraperitoneally implanted in seven mice of the physiological saline administration group. The frequency of abnormality indicates the rate of occurrence of BUN abnormality when 50 BUN or more is the abnormal level.

Example 2: Effect of MK on relieving hepatopathy

Wild mice with acute hepatopathy caused by carbon tetrachloride were prepared. The effects of administering MK on relieving acute hepatopathy were then monitored. Five mice were employed for each treatment. Once carbon tetrachloride diluted to 10% with food oil "Medium Chain Triglyceride: Panasate 800" (NOF corporation) were administered to the mice, they were fasted. Physiological saline,

0.017 mg of MK or 1.7mg of MK (JP-A No. Hei 9-95454), was administered intraperitoneally after 24 hours and again 8 hours after the first administration. Blood was collected after 16 hours, and serum GOT and GPT were measured (Figures 5 and 6).

The Student t test revealed that the GOT of the physiological saline group did not significantly differ from that of the MK (1.7 mg) administration group as shown in Figures 5 and 6. In contrast, GPT between the groups differed significantly at the 5% risk level. It was thus determined that MK significantly relieved acute hepatopathy caused by carbon tetrachloride.

Example 3: Effect of MK on relieving nephropathy

G401 cells derived from human infantile renal cancer (Wilms tumor) were used considering the side effects of antitumor agent cisplatin on kidneys.

G401 cells derived from human infantile renal cancer (Wilms tumor) were adjusted to 1 x $10^5$ cells/ml or 3 x $10^5$ cells/ml by 10% FBS/DME medium. 2 x $10^4$ cells or 6 x $10^4$ cells were inoculated per well of a 96-well plate (COSTAR: 3596) and incubated at 37℃ overnight under a 5% $CO_2$ atmosphere.

Subsequently, the cells were incubated in a 0.1% FBS/DME medium containing 2 μg/ml or 10 μg/ml of MK and in the same medium without MK as a control group for six hours. After the second incubation, the cells were incubated in a medium containing 10 μM of cisplatin (product name, briplatin, Bristol Myers Squibb Company) for 2 hours.

The cultured media were washed four times after the incubation, and the cell incubation was continued in a media containing 2 μg/ml or 10 μg/ml of MK the same as above.

Proliferation activity of the live cells was measured with Premix WST-1 Cell Proliferation Assay System (Takara) to evaluate the effect of MK on relieving nephropathy. Proliferation activity was assayed by intracellular division and proliferation of mitochondria reflected in absorbance (450 nm, control: 655 nm) in the same way as in an MTT assay.

Specifically, the cells were treated by cisplatin. Premix WST-1 reagent, up to 1/10 of the medium, was then added to each well on

the second, third, or fourth day after the cispaltin treatment, and the cells were incubated for 4 hours. The absorbance of each well was measured with a Plate Reader (BIO-RAD; Model 3550) (Figures 7 and 8). Figures 7 and 8 indicate that 2 to 10 μg/ml of MK dramatically decreased the number of G401 cell deaths due to cisplatin (antitumor agent) nearly two fold.

Example 4: Effect of PTN on relieving drug-induced nephropathy

ICR mice (male, 8 to 10 week-old) were divided into two groups; one for physiological saline administration and the other for PTN administration (11 mice each). The dosages of PTN and physiological saline were 500 μg/kg for each group (Merenmies, J. and H. Rauvala: J. Biol. Chem. 265: 16721-16724, 1990). Briplatin (Bristol Myers Squibb Company) was used as cisplatin.

PTN or physiological saline was administered intraperitoneally to each mouse of the above two groups for three days continuously. On the fourth day, whole blood was collected from three mice of each group, and serum was prepared to serve as the serum sample before the administration of briplatin.

In the afternoon of the same day (the fourth day), 15 mg/kg of briplatin was intraperitoneally administered to each of the rest of mice. PTN or physiological saline was continuously administered to the rest of mice on each day until the seventh day. On the sixth and eighth days, whole blood was collected from the mice, and the serum was prepared to serve as the serum sample on the second and fourth days after the administration of briplatin.

Blood urea nitrogen (BUN), a representative marker of renal function, (Figure 9) and serum creatinine (Figure 10) of each sample were measured by Iatro-chrom UN (IATRON LABORATORIES, INC.) and Creatinine-test Wako (Wako Pure Chemical Industries, Ltd.), respectively. Figures 9 and 10 show that BUN and creatinine in the physiological saline administration group tended to be higher than that in the PTN administration group. These results indicate that both PTN and MK relieve drug-induced nephropathy.

Industrial Applicability

The present invention demonstrates that the MK family proteins effectively relieve drug-induced nephropathy and hepatopathy. Therefore, pharmaceutical composition of the invention, comprising MK family protein as an effective ingredient, is useful for relieving nephropathy and hepatopathy induced by drugs, especially antitumor agents.

# CLAIMS

1. A pharmaceutical composition for treating or preventing drug-induced nephropathy or hepatopathy, comprising an MK family protein as an effective ingredient.

2. The pharmaceutical composition of claim 1, wherein said composition treats or prevents drug-induced nephropathy caused by the administration of an antitumor agent.

3. The pharmaceutical composition of claim 2, wherein said antitumor agent is cisplatin.

4. Use of an MK family protein for preparing a pharmaceutical composition for treating or preventing drug-induced nephropathy or hepatopathy.

5. The use of an MK family protein of claim 4, wherein said drug-induced nephropathy is caused by an antitumor agent.

6. The use of an MK family protein of claim 4, wherein said antitumor agent is cisplatin.

7. A method for relieving drug-induced nephropathy or hepatopathy, which comprises administering an MK family protein.

8. The method for relieving drug-induced nephropathy or hepatopathy of claim 7, wherein said drug-induced nephropathy is caused by the administration of an antitumor agent.

9. The method for relieving drug-induced nephropathy or hepatopathy of claim 7, wherein said antitumor agent is cisplatin.

# ABSTRACT

The present invention provides a novel drug for relieving drug-induced nephropathy and acute hepatopahy containing a midkine (MK) family protein such as pleiotrophin (PTN). The MK family proteins can inhibit nephropathy induced by an antitumor agent or acute hepatopathy caused by carbon tetrachloride and thus effectively relieve drug-induced nephropathy or hepatopathy.